INHIBITORS OF ABC DRUG TRANSPORTERS IN MULTIDRUG RESISTANT CANCER CELLS

BACKGROUND

5   [0001]   ATP-binding cassette (ABC) proteins play a central role in living cells through their role in nutrient uptake, protein, drug and antibiotic secretion, osmoregulation, antigen presentation, signal transduction and others. The majority of ABC proteins have a translocation function either in import of substrates or secretion of cellular products or xenobiotics.

10   [0002]   The ATP binding cassette (ABC) superfamily is one of the largest superfamilies known. With the multiplication of genome sequencing projects, new sequences appear every week in the GenBank database. Members of this family posses a highly conserved protein or module, the ABC module, that displays the WalkerA and WalkerB motifs separated by a short, highly conserved, sequence (consensus LSGGQ) called a signature sequence or linker peptide. Most ABC cassette proteins are primary transporters for unidirectional movement of molecules across biological membranes. The substrates handled by these transporters are extraordinarily varied ranging from small molecules to macromolecules.

[0003]   ABC cassette proteins of particular interest are the drug transporters associated with multidrug resistance in humans. The human multidrug resistance protein family currently has six well characterized members (Borst et al, J. Natl Cancer Inst. 92:1295-___ (2000)). Originally implicated in the resistance of tumor cells to chemotherapeutic agents, the multi-drug resistance protein MDR1, also known as P-glycoprotein (PGP), belongs to the ATP-binding cassette family of proteins. PGP is expressed in the human intestine,

25   blood brain barrier, liver, and other tissues. Expression of PGP, localized to cell membranes may affect the bioavailability of drug molecules that are substrates for this transporter. Drugs that inhibit P-glycoprotein can alter the absorption, disposition and elimination of co-administered drugs and can enhance bioavailability or cause unwanted drug-drug interactions. Interaction with PGP can be studied using either direct assays of

30   drug transport in polarized cell systems or with indirect assays such as drug-stimulated ATPase activity and inhibition of the transport of fluorescent substrates.

[0004]   P-glycoprotein is located in the apical surface of capillary endothelium in the brain. Knockout mice lacking the gene encoding P-glycoprotein show elevated brain

concentrations of multiple systemically administered drugs, including opioids as wells as chemotherapeutic agents. Chen and Pollack, J. Pharm. Exp. Ther. 287:545-552 (1998) and Thompson, et al., Anesthesiology 92:1392-1299 (2000).

[0005]   Opioid receptor antagonists are generally accepted for use in the treatment of human conditions of ailments for reversing opioid toxicity and overdoses, and in preventing abuse of opioid receptor agonists, such as heroin or morphine. For these uses, the antagonists such as naloxone or naltrexone is used in relatively high concentrations in order to effectively block the activity and/or effects of the opioid receptor agonist by antagonizing the opioid receptor agonist at opioid receptors on nociceptive neurons.

[0006]   Thus, a continuing need exists for methods to increase the ability of clinicians administer bioactive substances across the blood brain barrier.

[0007]   ABC cassette proteins have also been implicated in the resistance of many human cancers to traditional chemotherapeutic agents, i.e., multidrug resistance. The major documented cause of multidrug resistance of cancers is the overexpression of P-glycoprotein, which is capable of pumping structurally diverse antitumor drugs from cells. See D. Houseman et al., A Molecular Genetic Approach to the Problem of Drug Resistance in Chemotherapy, 504-517 (1987) (Academic Press, Inc.); R. Fine and B. Chabner, Multidrug Resistance, in Cancer Chemotherapy 8, 117-128 (H. Pinedo and B. Chabner eds. 1986); Ann Rev. Biochem 58:137-171 (1989). Increased expression of the gene encoding P-glycoprotein (mdr) is found in many malignant cells, including leukemias, lymphomas, sarcomas and carcinomas, and may be upregulated by the onset of a malignancy and/or cellular contact with chemotherapeutic agents. Once active, P-glycoprotein is believed to function as a "hydrophobic vacuum cleaner" which expels hydrophobic drugs from targeted cells. Such drugs include many anti-cancer drugs and cytotoxic agents, such as vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes, actinomycins, colchicine, puromycin, toxic peptides (e.g., valinomycin), topotecan, and ethidium bromide. See I. Pastan and M. Gottesman, New England J. Med. 1388, 1389 Table 1 (May 28, 1987).

[0008]   Tumor cells expressing elevated levels of the multiple drug transporter accumulate far less antitumor agents intracellularly than tumor cells having low levels of this enzyme. The degree of resistance of certain tumor cells has been documented to correlate with both elevated expression of the drug transporter and reduced accumulation of antitumor drugs. See M. Gottesman and I. Pastan, J. Biol. Chem. 263, 12163 (1988); see also A. Fojo et al., Cancer Res. 45, 3002 (1985).

[0009]   Reduced intracellular levels of antitumor agents in the tumor suppresses chemotherapeutic efficacy. Tumors having elevated levels of the multiple drug transporter require therapeutic doses of cancer suppressants far in excess of tumors exhibiting lower levels of drug transporters. Agents that inhibit the active efflux of antitumor agents by the drug transporter or agents that potentiate the efficacy of chemotherapeutic agents would enhance the activity of various antitumor agents on tumor cells.

[00010]   Thus, a continuing need exists for methods to combat multidrug resistance in cancers. Inhibition of PGP function in PGP-mediated multidrug resistance has been shown to lead to a net accumulation of anti-cancer agent in the cells. For example, verapamil a known calcium channel blocker was shown to sensitize MDR cells to vinca alkaloids in vitro and in vivo: Cancer Res., 41, 1967-1972 (1981).

SUMMARY OF THE INVENTION

[00011]   The present invention provides methods of increasing efficacy of an anti-tumor agent by co-administering to patient suffering from a multidrug resistant cancer a dose of an anti-tumor agent and a dose of an opioid inhibitor of the ABC drug transporter. The anti-tumor agent is a substrate of an ABC drug transporter and the dose of the opioid inhibitor of the ABC drug transporter is sufficient to reduce efflux of the anti-tumor agent from the microbe.

[00012]   Further the invention provides for identification of inhibitors of ABC drug transporters having a pharmacophore defined by a hydrogen bonding moiety at a three-dimensional location corresponding to the hydroxyl at position 3 of naltrexone, a hydrogen bonding moiety at a three-dimensional location corresponding to the hydroxyl at position 14 of naltrexone, a hydrophobic moiety at a three-dimensional location corresponding to the cyclopropyl moiety appended to the nitrogen of naltrexone, and a region of electron density at a three-dimensional location corresponding to the ethylene moiety at 6-position of naltrexone.

[00013]   The invention provides methods of decreasing toxicity associated with treating a cancer patient by co-administering a sub-therapeutic dose of an anti-tumor agent and a dose of an opioid inhibitor of a drug transporter protein. The dose of opioid inhibitor is sufficient to increase the concentration of the anti-tumor agent within the cancer cell and further is sufficient to inhibit growth of the cancer.

[00014]   The invention also provides compositions for treating multidrug resistant cancer cells with a combination of an anti-tumor agent and an opioid inhibitor of a ABC drug transporter. The anti-tumor agent is a substrate of the ABC drug transporter.

[00015]   Another aspect of the invention is methods of enhancing the anti-tumor activity of an anti-tumor agent against a cancer cell by contacting the cancer cell with the anti-tumor agent and an opioid inhibitor of an ABC drug transporter in an amount effective to inhibit a drug transporter in the cancer cell. The cancer cell expresses an ABC drug transporter and the anti-tumor agent is a substrate of the ABC drug transporter.

[00016]   The invention provides methods of suppressing growth of a cancer cell expressing an ABC drug transporter protein by contacting the cancer cell with a sub-therapeutic amount of an anti-tumor agent in the presence of an opioid inhibitor of the ABC drug transporter.

[00017]   The invention also provide methods of inhibiting a P-glycoprotein in a patient suffering from cancer. A P-glycoprotein inhibiting amount of naltrexone, naloxone or nalmefene is administered to the patient before, with, or after the administration to the patient of a therapeutic or sub-therapeutic amount of an anti-tumor agent.

[00018]   In another aspect, the invention provides methods of identifying compounds for improved treatment of cancer. The method includes identifying an anti-tumor agent, assaying the ability of the anti-tumor agent to be transported across a membrane by an ABC protein, and repeating the transport assay to determine whether addition of an opioid inhibitor of an ABC drug transporter inhibits transport of the anti-tumor agent across the membrane. The desired compound is identified as a compound that is transported by an ABC protein and whose ABC protein-mediated transport is inhibited by an opioid inhibitor.

[00019]   The invention provides methods for screening for an opioid inhibitor of an ABC drug transporter by determining whether a potential opioid inhibitor inhibits growth of a cancer cell in the presence of sub-therapeutic amount of anti-microbial agent. Inhibition of growth is assayed by comparing the growth of a cancer cell which expresses the ABC drug transporter, with growth of a second cancer cell which does not produce the ABC drug transporter. Both are grown in the presence of the sub-therapeutic amount of the anti-tumor agent.

[00020]   The invention also provides methods for screening for an opioid inhibitor of an ABC drug transporter. The method includes contacting a potential opioid inhibitor of an ABC drug transporter protein with the ABC drug transporter protein in the presence of a

compound selected from the group consisting of naltrexone, naloxone and nalmefene,
wherein the compound is detectably labeled and measuring the amount of detectably labeled
compound bound to the ABC drug transporter. The measured amount is compared the to
the amount of detectably labeled compound bound by the ABC drug transporter when the
drug transporter is contacted with the compound alone. An ABC drug transporter inhibitor
is identified by a decreased amount of labeled compound bound to the ABC drug
transporter when the potential inhibitor is present.

[00021]   The invention also provides methods of treating cancer in an animal, by
administering an anti-tumor agent and an amount of naltrexone, naloxone or nalmefene
sufficient to increase the intracellular concentration of the anti-tumor agent. The ABC drug
transporter inhibitor increases the susceptibility of the cancer cell to the anti-tumor agent.

[00022]   Finally, the invention provides ABC drug transporter inhibitors of the formula:
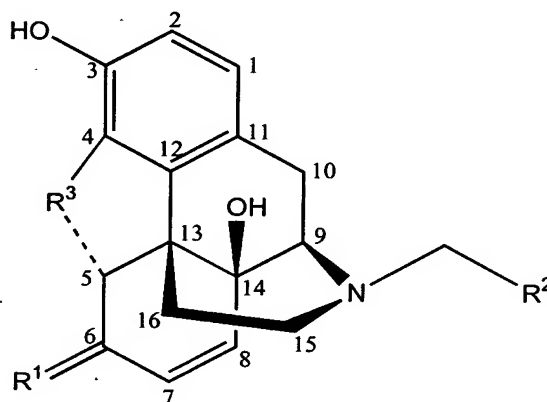
wherein R^1 is CH_2 or O;

wherein R^2 is a cycloalkyl, unsubstituted aromatic, alkyl or alkenyl; and

wherein R^3 is O, CH_2 or NH.

BRIEF DESCRIPTION OF THE DRAWINGS

[00023]   Fig. 1 illustrates the chemical structures of naltrexone, naloxone, nalmefene, 6-β-naltrexol and nalorphine.

[00024]   Fig. 2 presents an overlay of the opioid analogues, naltrexone, naloxone, nalmefene, 6-β-naltrexol and nalorphine.

[00025]   Fig. 3A shows the molecular orbitals and electrostatic potential of nalmefene as calculated using Spartan (Wavefunction, Inc.).

[00026] Fig. 3B shows the molecular orbitals and electrostatic potential of naloxone as calculated using Spartan (Wavefunction, Inc.).

[00027] Fig 4A-4AH provide information about the 200 nearest neighbors to the opioid analogues examined in the QSAR analysis.

DETAILED DESCRIPTION

[00028] The present invention is based in part on surprising results from transport studies that compounds previously identified as opioid receptor antagonists are inhibitors of ABC drug transporter proteins, a prototypical such as the exemplary P-glycoprotein, PGP-1a. Administration of opioid receptor antagonists, such as naloxone, nalmefene and naltrexone, unexpectedly result in increased intracellular concentrations of co-administered therapeutic agents in cells expressing an ABC drug transporter protein, particularly in multidrug resistant cancer cells expressing PGP1a. The present invention provides a novel class of drug transporter inhibitors that act by inhibiting ABC transporter proteins and their associated ATPase as described herein and further provides a pharmacophone that identifies new drug targets that are inhibitors of ABC transporter proteins. As used herein, the terms "transporter" and "drug transporter" refer to a protein for the carrier-mediated influx and efflux of drugs and endocytosis of biologically active molecules across a cell membrane barrier, including across a gut, liver, or blood-brain barrier. An inhibitor of a transporter is expected to increase the efficacy of an active agent according to the invention, wherein the transporter inhibitor reduces efflux across the cellular membrane of a cancer cell and/or increases influx into the cancer cell, thereby enhancing the therapeutic effectiveness of the active agent. Preferably the drug transporter protein is a member of the ABC superfamily, referred to as an "ABC drug transporter." The ABC drug transporter may either be a multidrug resistance protein (MDR) or a multidrug resistance-associated protein (MRP).

[00029] Among the ABC superfamily of drug transporters, there are several closely conserved regions, the nucleotide binding motifs of the WalkerA region and WalkerB region, and the short consensus sequence (leucine-serine-glycine-glycine-glutamine, or LSGGQ) . Essentially every ABC drug transporter contains the consensus sequence or a very closely related sequence. The QSAR analysis of the present invention provides the very surprising result that the opioid receptor antagonists that act as ABC drug transporter inhibitors bind to this LSGGQ consensus sequence. Thus the present invention defines a strictly conserved inhibition site shared among all ABC drug transporter proteins. Therefore, the ABC drug transporter inhibitor, including compounds identified as opioid

receptor antagonists, according to the present invention will function as an inhibitor of a ABC drug transporter protein that shares the LSGGQ conserved sequence.

[00030]   Thus, the present invention is based up the identification of a new class of drug transporter inhibitors. The term "drug transporter inhibitor" or "ABC drug transporter inhibitor refers to a compound that binds to an ABC drug transporter protein and inhibits, *i.e.*, either completely blocks or merely slows, transport of compounds across biological barriers. Drugs that inhibit drug transporters can alter the absorption, disposition and elimination of co-administered drugs and can enhance bioavailability or cause unwanted drug-drug interactions. Interaction with drug transporters can be studied using either direct assays of drug transport in polarized cell systems or with indirect assays such as drug-stimulated ATPase activity and inhibition of the transport of fluorescent substrates. Drugs affected by the drug transporter, P-glycoprotein, include ondasetron, dexamethasone, domperidone, loperamide, doxorubicin, neifinavir, indinevir, sugguinavir, erythromycin, digoxin, vinblastine, paclitaxel, invermectin and cyclosporin. Known inhibitors of P-glycoprotein include ketoconazole, verapamil, quinidine, cyclosporin, digoxin, erythromycin and loperamide. See, *e.g.*, Intl. J. Clin. Pharmacol. Ther. 38:69-74 (1999). The present invention unexpectedly identifies opioid receptor antagonists, such as naloxone, naltrexone and nalmefene, as potent inhibitors of the drug transporter, P-glycoprotein. The QSAR analysis of the invention demonstrates that the opioid receptor antagonists are also inhibitors of ABC drug transporters, especially of microbial homologues of human PGP1a.

[00031]   An "opioid receptor antagonist" is an opioid compound or composition including any active metabolite of such compound or composition that in a sufficient amount attenuates (*e.g.*, blocks, inhibits, prevents or competes with) the action of an opioid receptor agonist. An opioid receptor antagonist binds to and blocks (*e.g.*, inhibits) opioid receptors on nociceptive neurons. Opioid receptor antagonists include: naltrexone (marketed in 50mg dosage forms as ReVia® or Trexan®), nalaxone (marketed as Narcan®), nalmefene, methylnaltrexone, naloxone, methiodide, nalorphine, naloxonazine, nalide, nalmexone, nalbuphine, nalorphine dinicotinate, naltrindole (NTI), naltrindole isothiocyanate (NTII), naltriben (NTB), nor-binaltorphimine (nor-BNI), b-funaltrexamine (b-FNA), BNTX, cyprodime, ICI-174,864, LY117413, MR2266, or an opioid receptor antagonist having the same pentacyclic nucleus as nelmefene, naltrexone, nalorphine, nalbuphine, thebaine, levallorphan, oxymorphone, butorphanol, buprenorphine, levorphanol meptazinol, pentazocine, dezocine, or their pharmacologically effective esters or salts. In some

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preferred embodiments, the opioid receptor antagonist is naltrexone, nalmefene, naloxone, or mixtures thereof.

[00032]   The term "opioid" refers to compounds which bind to specific opioid receptors and have agonist (activation) or antagonist (inactivation) effects at these receptors, and thus are "opioid receptor agonists" or "opioid receptor antagonists."

[00033]   In particular, the present invention contemplates enhancing the efficacy of antitumor agents by co-administering the antitumor agent with an ABC transporter inhibitor such as an opioid receptor antagonist. The opioid receptor antagonists, naltrexone, naloxone and nalmefene, are particularly suited for the present invention. Although some inhibitors of ABC drug transporters are known in the art, many of these are extremely toxic, especially if used repeatedly over a period of time. For example, when used orally, ketoconazole has been associated with hepatic toxicity, including some fatalities. The opioid receptor antagonists, however, historically have limited side effects, particularly at the low concentrations administered in the present invention.   Each of the antagonists naltrexone, naloxone and nalmefene have been approved by the FDA for use in antagonistically effective amounts for treatment of opioid overdose and addictions.

[00034]   Co-administration of an ABC drug transporter inhibitor and an antitumor agent is expected to provide more effective treatment of cancer. Concurrent administration of the two agents may provide greater therapeutic effects *in vivo* than the antitumor agent provides when administered singly. For example, concurrent administration may permit a reduction in the dosage of the antitumor agent with achievement of a similar therapeutic effect. Alternatively, the concurrent administration may produce a more rapid or complete antitumor effect than could be achieved with the antitumor agent alone.

[00035]   "Co-administer," "co-administration," "concurrent administration" or "co-treatment" refers to administration of an antitumor agent and a drug transporter inhibitor, in conjunction or combination, together, or before or after each other. The antitumor agent and the drug transporter inhibitor may be administered by different routes. For example, the antitumor agent may be administered orally and the drug transporter inhibitor intravenously, or vice versa. The antitumor agent and the drug transporter inhibitor are preferably both administered orally, as immediate or sustained release formulations. The antitumor agent and drug transporter inhibitor may be administered simultaneously or sequentially, as long as they are given in a manner to allow both agents to achieve effective concentrations to yield their desired therapeutic effects.

[00036]   "Therapeutic effect" or "therapeutically effective" refers to an effect or effectiveness that is desirable and that is an intended effect associated with the administration of an active agent according to the invention. A "therapeutic amount" is the amount of an active agent sufficient to provide a therapeutic effect. "Sub-therapeutic

5   amount" is an amount of the active agent which does not cause a therapeutic effect in a patient administered the active agent alone, but when used in combination with a drug transporter inhibitor is therapeutically effective.

[00037]   Therapeutic effectiveness is based on a successful clinical outcome, and does not require that the antitumor agent or agents kill 100% of the cancer cells. Success depends on

10   achieving a level of antitumor activity at the site of the cancer that is sufficient to inhibit the cancer cells in a manner that tips the balance in favor of the host. When host defenses are maximally effective, the antitumor effect required may be minimal.

Drug resistance

[00038]   The term "drug resistance" refers to the circumstance when a disease does not

15   respond to a treatment drug. Drug resistance can be either intrinsic or acquired. "Multidrug resistance" means a specific type of drug resistance characterized by cross-resistance of a disease to more than one functionally and/or structurally unrelated drugs. The term "ABC transporter-mediated multidrug resistance" refers to multidrug resistance due to the activity of an ABC drug transporter protein.

20   [00039]   One of the major problems of cancer chemotherapy is the existence of drug resistance in tumors resulting in reduced responsiveness to chemotherapy. Some human cancers, e.g. kidney and colon carcinoma, are drug resistant before treatment begins, while in others drug resistance develops over successive rounds of chemotherapy. One type of drug resistance, called multidrug resistance, is characterized by cross resistance to

25   functionally and structurally unrelated drugs. Typical drugs that are affected by the multidrug resistance are doxorubicin, vincristine, vinblastine, colchicine and actinomycin D, and others. At least some multidrug resistance is a complex phenotype which has been linked to a high expression of a cell membrane drug efflux transporter called Mdr1 protein, also known as P-glycoprotein. This membrane "pump" has broad specificity and acts to

30   remove from the cell a wide variety of chemically unrelated toxins. (See Endicott, J. A., et al. "The Biochemistry of P-Glycoprotein-Mediated Multidrug Resistance", Ann. Rev. Biochem. Vol. 58, pgs. 127-71, 1989.)

[00040]   Cancer chemotherapy with cytotoxic agents can be successful only if the tumor cells are more sensitive than normal cells whose destruction is incompatible with survival of the host. Success, defined either as cure or clinically significant remission, is not readily explained by the still popular idea that tumor cells are more susceptible to cytotoxic agents because they are dividing more rapidly than vital normal cells, e.g. hematopoietic precursor cells. That rapid proliferation does not wholly account for the selective drug sensitivity of tumors is demonstrated by the common observations that some drug-sensitive cancers are not rapidly dividing, and that many rapidly proliferating tumors exhibit resistance. To say that the mechanisms accounting for the success or failure of chemotherapy for most human tumors is incompletely understood today is undoubtedly an understatement.

[00041]   However, recent evidence suggests that the selectivity of chemotherapy for the relatively few tumors ever cured by drugs depends, to a large extent, upon their easy susceptibility to undergo apoptosis, i.e. to kill themselves. Many cytotoxic drugs that kill cells by crippling cellular metabolism at high concentration can trigger apoptosis in susceptible cells at much lower concentration. This appears to account for the unusual chemosensitivity of many lymphoid tumors, since many normal lymphocytes are "primed" to undergo self destruction as an essential part of the mechanism for generating and controlling diversity of the immune response. Increased susceptibility to apoptosis may also be acquired by tumor cells as a byproduct of the genetic changes responsible for malignant transformation. For example, tumor cells with constitutive c-myc expression may undergo apoptosis in response to DNA damage by anticancer agents, whereas normal cells are able to pause at checkpoints in the cell cycle to repair the damage, or may not be cycling at all, rendering them highly resistant to apoptosis in this setting.

[00042]   Antitumor agent from a number of classes of compounds can be co-administered with an opioid inhibitor of an ABC drug transporter protein. Preferably, the antitumor agent is selected from the following classes of compounds: Alkylating Agents, such as nitrogen mustards, ethyleneimines, methylamelamines, alkyl sulfonates, nitrosoureas, or triazene, Antimetabolites, such as folic acid analogs, pyrimidine analogs, purine analogs, Vinca alkaloids, taxanes, epipodophyllotoxins, Anthracyclines, Antiproliferative agents, Tubulin Binding agents, Enediynes, anthracededione, substituted urea, methylhydrazine derivatives, the Pteridine family of drugs, Taxanes, , Dolastatins, Topoiosomerase inhibitors, Mytansinoids, and Platinum coordination complexes.

[00043]   Particularly, the antitumor agent is advantageously selected from the following compounds or a derivative or analog thereof: Doxorubicin, Daunorubicin, Vinblastine, Vincristine, Calicheamicin, Etoposide, Etoposide phosphate, CC-1065, Duocarmycin, KW-2189, Methotrexate, Methopterin, Aminopterin, Dichloromethotrexate, Docetaxel, Paclitaxel, Epithiolone, Combretastatin, Combretastatin A4 Phosphate, Dolastatin 10, Dolastatin 11, Dolastatin 15, Topotecan, Camptothecin, Mitomycin C, Porfiromycin, 5-Fluorouracil, 6-Mercaptopurine, Fludarabine, Tamoxifen, Cytosine arabinoside, Adenosine Arabinoside, Colchicine, Carboplatin, Mitomycin C, Bleomycin, Melphalan, Cyclosporin A, Chloroquine, Maytansine or Cisplatin. By derivative is intended a compound that results from reacting the named compound with another chemical moiety, and includes a pharmaceutically acceptable salt, acid, base or ester of the named compound. By analog is intended a compound having similar structural and functional properties, such as biological activities, to the named compound.

[00044]   For administration to human subjects or in the treatment of any clinical conditions, the pharmaceutical compositions or dosage forms of this invention may be utilized in compositions such as capsules, tablets or pills for oral administration, suppositories for rectal administration, liquid compositions for parenteral administration and the like.

[00045]   The pharmaceutical compositions or dosage forms of this invention may be used in the form of a pharmaceutical preparation, for example, in solid or semisolid form, which contains one or more of the drug transporter inhibitors, as an active ingredient, alone, or in combination with one or more therapeutic agents. Any drug transporter inhibitor or therapeutic agent may be in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral applications. The drug transporter inhibitor may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for capsules, tablets, pellets, suppositories, and any other form suitable for use. The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium, trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid or semisolid form, and in addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The drug transporter inhibitor, alone or in conjunction with a therapeutic agent, is included in the pharmaceutical composition or dosage form in an amount sufficient to produce the

desired effect upon the process or condition, including a variety of conditions and diseases in humans.

[00046]   For preparing solid compositions such as tablets, the drug transporter inhibitor, alone or in conjunction with therapeutic agent, is mixed with a pharmaceutical carrier, e.g., conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical diluents, e.g., water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a non-toxic pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the drug transporter inhibitor, alone or in conjunction with therapeutic agent, is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as capsules, tablets, caplets, or pills. The capsules, tablets, caplets, or pills of the novel pharmaceutical composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate. Controlled release (e.g., slow-release or sustained-release) dosage forms, as well as immediate release dosage forms are specifically contemplated according to the present invention.

[00047]   Compositions in liquid forms in which a therapeutic agent may be incorporated for administration orally or by injection include aqueous solution, suitable flavored syrups, aqueous or oil suspensions, and emulsions with acceptable oils such as cottonseed oil, sesame oil, coconut oil or peanut oil, or with a solubilizing or emulsifying agent suitable for intravenous use, as well as elixirs and similar pharmaceutical vehicles. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone or gelatin.

[00048]   Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders.

The liquid or solid compositions may contain suitable pharmaceutical 1v acceptable excipients as set out above. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably sterile pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be breathed directly from the nebulizing device or the nebulizing device may be attached to a face mask, tent or intermittent positive pressure breathing machine. Solution, suspension or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

[00049] A drug transporter inhibitor alone, or in combination with a therapeutic agent, may be administered to the human subject by known procedures including but not limited to oral, sublingual, intramuscular, subcutaneous, intravenous, intratracheal, transmucosal, or transdermal modes of administration. When a combination of these compounds are administered, they may be administered together in the same composition, or may be administered in separate compositions. If the therapeutic agent and the drug transporter inhibitor are administered in separate compositions, they may be administered by similar or different modes of administration, or may be administered simultaneously with one another, or shortly before or after the other.

[00050] The drug transporter inhibitors alone, or in combination with therapeutic agents are formulated in compositions with a pharmaceutically acceptable carrier ("pharmaceutical compositions"). The carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Examples of suitable pharmaceutical carriers include lactose, sucrose, starch, talc, magnesium stearate, crystalline cellulose, methyl cellulose, carboxymethyl cellulose, glycerin, sodium alginate, gum arabic, powders, saline, water, among others. The formulations may conveniently be presented in unit dosage and may be prepared by methods well-known in the pharmaceutical art, by bringing the active compound into association with a carrier or diluent, or optionally with one or more accessory ingredients, e.g., buffers, flavoring agents, surface active agents, or the like. The choice of carrier will depend upon the route of administration. The pharmaceutical compositions may be administered as solid or semisolid formulations, including as capsules, tablets, caplets, pills or patches. Formulations may be presented as an immediate-release or as a controlled-release (e.g., slow-release or sustained-release) formulation.

[00051]   For oral or sublingual administration, the formulation may be presented as capsules, tablets, caplets, powders, granules or a suspension, with conventional additives such as lactose, mannitol, corn starch or potato starch; with binders such as crystalline cellulose, cellulose derivatives, acacia, corn starch, gelatins, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes, or the like; with disintegrators such as corn starch, pbtato starch, methyl cellulose, agar, bentonite, xanthan gums, sodium carboxymethyl-cellulose or the like; or with lubricants such as talc, sodium oleate,. sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride or the like.

[00052]   For transdermal administration, the compounds may be combined with skin penetration enhancers such as propylene glycol, polyethylene glycol, isopropanol, ethanol, oleic acid, N-methylpyrrolidone, or the like, which increase the permeability of the skin to the compounds, and permit the compounds to penetrate through the skin and into the bloodstream. The compound/enhancer compositions also may be combined additionally with a polymeric substance such as ethylcellulose, hydroxypropyl cellulose, ethylene/ vinylacetate, polyvinyl pyrrolidone, or the like, to provide the composition in gel form, which can be dissolved in solvent such as methylene chloride, evaporated to the desired viscosity, and then applied to backing material to provide a patch.

[00053]   For intravenous, intramuscular, or subcutaneous administration, the compounds may combined with a sterile aqueous solution which is preferably isotonic with the blood of the recipient. Such formulations may be prepared by dissolving solid active ingredient in water containing physiologically compatible substances such as sodium chloride, glycine, or the like, and/or having a buffered pH compatible with physiological conditions to produce an aqueous solution, and/or rendering said solution sterile. The formulations may be present in unit or multi-dose containers such as sealed ampoules or vials.

[00054]   When the drug transporter inhibitor is used in combination with the therapeutic agent, the amount of the therapeutic agent administered may be a therapeutic or sub-therapeutic amount. As used herein, a "therapeutic" amount is the amount of the therapeutic agent which causes a therapeutic effect in a subject administered the therapeutic agent alone. The amount of the drug transporter inhibitor may be an amount effective to enhance the therapeutic potency of and/or attenuate the adverse side effects of the therapeutic agent. The optimum amounts of the drug transporter inhibitor administered

alone or in combination with a therapeutic agent will of course depend upon the particular drug transporter inhibitor and therapeutic agent used, the carrier chosen, the route of administration, and/or the pharmacokinetic properties of the subject being treated.

[00055]   When the drug transporter inhibitor is administered alone, the amount of the drug transporter inhibitor administered is an amount effective to enhance or maintain the therapeutic potency of the therapeutic agent and/or attenuate or maintain the adverse side effects of the therapeutic agent. This amount is readily determinable by one skilled in the art according to the invention.

[00056]   The present invention is described in the following examples which are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the invention as defined in the claims which follow thereafter.

EXAMPLES

Example 1 – Opioid Receptor Antagonists Inhibit Human PGP-Mediated Transport

[00057]   Porcine kidney-derived, LLC-PK$_1$, cells expressing human PGP cDNA (designated 15B-J) were cultured in 24 well Transwell™ culture inserts at 37° C on an orbital shaker. Transport assays were conducted in 24 well Transwell™ culture inserts with Hanks Balanced Salt Solution (HBSS) buffered with the addition of 10 mM HEPES (pH 7.2).

[00058]   The test substances, naloxone, naltrexone and nalmefene, were purchased from Sigma-Aldrich. Stock solutions of the compounds were made in DMSO, and dilutions of these in transport buffer were prepared for assay in the monolayers. The DMSO concentration (0.55%) was constant for all conditions within the experiment. All test substance and control drug solutions prepared in HBSS/HEPES buffer contained 0.55% DMSO.

[00059]   The test substance was added to the donor and receiver chambers. Duplicate monolayers and thirteen test substance concentrations of 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 and 100 µM were used. PGP substrate [^{3}H]-digoxin, at 5 µM was added to the donor chamber (either the apical or basolateral chamber depending on the direction of transport). After an incubation time of 90 minutes, a sample from the receiver chamber was analyzed for the amount of digoxin present. The positive control for inhibition was 25 µM ketoconazole added to donor and receiver chambers with 5 µM [^{3}H]-digoxin added to the donor chamber. The negative control for inhibition was 5 µM [^{3}H]-

digoxin added to the donor chamber (either the apical or basolateral chamber depending on the direction of transport) with Hanks Balanced Salt Solution (HBSS) buffered with the addition of 10 mM HEPES (pH 7.2) and DMSO at 0.55% in the receiver chamber.

[00060]   The rate of digoxin transported from the apical chamber to the basolateral chamber (A to B) and from the basolateral chamber to the apical chamber (B to A) was measured and apparent permeability P_{app} constants calculated. The polarization ratio $P_{app\ B\ to\ A}/P_{app\ A}$ was calculated. A lower polarization ratio in the 15B-J cells with test substance relative to that without test substance provides evidence for inhibition of PGP-mediated digoxin transport by the test substance. Transport of 5 µM [3H]-digoxin was measured following coincubation with the test substances at nominal concentrations in the range of 0 to 100 µM. Inhibition of digoxin transport was calculated by comparison of the digoxin polarization ratio in the presence of the test substance, to the ratio in the absence of test substance. The positive control for inhibition was 25 µM ketoconazole coincubated with digoxin. The inhibition of PGP-mediated transport in human PGP-expressing porcine kidney cell monolayers by naloxone is summarized in Table 1.

Table 1: Naloxone inhibition of PGP-mediated transport

Naloxone Concentration (µM)		Digoxin Polarization Ratio (B-A/A-B)	% Inhibition of Digoxin Transport	Ketoconazole Normalized % Inhibition of Digoxin Transport
nominal	measured			
0	-	3.7	-	-
0.0001	0.000021	3.5	4.4	6.2
0.0003	0.000138	3.5	6.0	8.4
0.001	0.00085	3.4	7.3	10
0.03	0.0021	3.6	4.0	5.7
0.01	0.0083	3.8	-3.2	-4.5
0.03	0.021	3.5	4.1	5.7
0.1	0.074	3.8	-1.9	-2.7
0.3	0.264	3.3	11.9	17
1	1.04	3.5	5.5	7.8

[00061]   The inhibition of PGP-mediated transport in human PGP-expressing porcine kidney cell monolayers by naltrexone is summarized in Table 2.

Table 2: Naltrexone inhibition of PGP-mediated transport

Concentration Naltrexone (µM)	Polarization ratio (B-A/A-B)	% Inhibition of Digoxin	Ketoconazole Normalized %

		Transport	Inhibition of Digoxin Transport
0	4.0	-	-
0.0001	3.6	10	
0.0003	3.5	14	
0.001	3.6	10	
0.003	3.7	8	
0.01	3.5	11	
0.03	3.8	5	
0.1	3.5	14	
0.3	3.3	18	
1.0	3.4	14	

[00062]   The inhibition of PGP-mediated transport in human PGP-expressing porcine kidney cell monolayers by nalmefene is summarized in Table 3.

Table 3: Nalmefene inhibition of PGP-mediated transport

Concentration Nalmefene (μM)	Polarization Ratio (B-A/A-B)	% Inhibition of Digoxin Transport	Ketoconazole Normalized % Inhibition of Digoxin Transport
0	4.5	-	-
0.0001	4.3	5.2	
0.0003	4.2	7.2	
0.001	4.4	2.8	
0.003	4.3	5.1	
0.01	4.3	3.9	
0.03	4.8	-7.2	
0.1	4.5	-0.3	
0.3	4.8	-5.6	
1.0	4.6	-2.6	

[00063]   Naloxone and naltrexone exhibited inhibitory behavior at the 30 and 100 μM concentrations. Digoxin transport appears to have been slightly inhibited at naloxone and

naltrexone concentrations below 30 μM, however the inhibition was not concentration-dependent. Digoxin transport was increasingly inhibited in response to increasing concentration of nalmefene at concentrations between 3 and 100 μM. The positive control, 25 μM ketoconazole, inhibited digoxin transport within the accepted range, indicating that the cell model performed as expected.

Example 2: 6-β-Naltrexol Does Not Inhibit Human PGP-Mediated Transport

[00064]   Porcine kidney-derived, LLC-PK$_1$, cells expressing human PGP cDNA (designated 15B-J) were cultured in 24 well Transwell™ culture inserts at 37° C on an orbital shaker. Transport assays were conducted in 24 well Transwell™ culture inserts with Hanks Balanced Salt Solution (HBSS) buffered with the addition of 10 mM HEPES (pH 7.2).

[00065]   The test substance, 6-β-naltrexol, was provided by LC Resources, Inc.,. Stock solutions of the compounds were made in DMSO, and dilutions of these in transport buffer were prepared for assay in the monolayers. The DMSO concentration (0.55%) was constant for all conditions within the experiment. All test substance and control drug solutions prepared in HBSS/HEPES buffer contained 0.55% DMSO.

[00066]   The test substance was added to the donor and receiver chambers. Duplicate monolayers and thirteen test substance concentrations of 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μM, were used. PGP substrate [^{3}H]-digoxin, at 5 μM was added to the donor chamber (either the apical or basolateral chamber depending on the direction of transport). After an incubation time of 90 minutes, a sample from the receiver chamber was analyzed for the amount of digoxin present. The positive control for inhibition was 25 μM ketoconazole added to donor and receiver chambers with 5 μM [^{3}H]-digoxin added to the donor chamber. The negative control for inhibition was 5 μM [^{3}H]-digoxin added to the donor chamber (either the apical or basolateral chamber depending on the direction of transport) and Hanks Balanced Salt Solution (HBSS) buffered with the addition of 10 mM HEPES (pH 7.2) and DMSO at 0.55% in the receiver chamber.

[00067]   Transport of 5 μM [^{3}H]-digoxin was measured following coincubation with test substance 6-β-naltrexol, at nominal concentrations in the range of 0 to 100 μM. Inhibition of digoxin transport was calculated by comparison of the digoxin polarization ratio in the presence of the test substance, to the ratio in the absence of test substance. The positive control for inhibition was 25 μM ketoconazole coincubated with digoxin. was slightly inhibited (mean of 8.5 +/- 7.1%) by 6-β-naltrexol in the concentration range of 0.0001 to 30

μM (Table 4 The inhibition did not appear to be concentration-dependent. At 100 μM 6-β-naltrexol, however, digoxin transport was more strongly inhibited (28%). The positive control, 25 μM ketoconazole, inhibited digoxin transport within the accepted range, indicating that the cell model performed as expected.

Table 4: 6-β-naltrexol inhibition of PGP-mediated transport

Nominal concentration of 6-β-naltrexol	Polarization Ratio (B-A/A-B)	% Inhibition of Digoxin Transport
0	4.7	-
0.0001	4.4	6.4
0.0003	4.7	0
0.001	4.8	-2.1
0.003	4.7	0
0.01	4.6	2.1
0.03	4.2	11
0.1	3.8	19
0.3	4.3	9
1.0	4.0	15
3.0	4.2	11
10	4.0	15
30	4.0	15
100	3.4	28
25μM Ketoconazole	1.0	79

[00068]   Digoxin efflux in the human PGP-expressing cell monolayers The test substance 6-β-naltrexol was not a potent inhibitor of PGP-mediated digoxin transport, in the concentration range tested.

Example 3 – Opioid Receptor Antagonists Inhibit PGP ATPase Activity

[00069]   The test substances, naloxone, naltrexone and nalmefene, were purchased from Sigma-Aldrich. Stock solutions of the compounds were made in DMSO, and dilutions of these in transport buffer were prepared for assay in the monolayers. The DMSO concentration (0.55%) was constant for all conditions within the experiment. All test

substance and control drug solutions prepared in HBSS/HEPES buffer contained 0.55% DMSO.

[00070]   The test substances were incubated in the membranes and supplemented with MgATP, with and without sodium orthovanadate present. Orthovanadate inhibits PGP by trapping MgADP in the nucleotide binding site. Thus, the ATPase activity measured in the presence of orthovanadate represents non-PGP ATPase activity and was subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity.

[00071]   ATPase assays were conducted in 96-well microtiter plates. A 0.06 ml reaction mixture containing 40 μg PGP membranes, test substance, and 4 mM MgATP, in buffer containing 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide, plus organic solvent was incubated at 37°C for 20 minutes. Triplicate incubations of ten test substance concentrations (of 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 and 100 μM) and the test vehicle without drug, were used. Identical reaction mixtures containing 100 μM sodium orthovanadate were assayed in parallel. The reactions were stopped by the addition of 30 μl of 10% SDS + Antifoam A. The incubations were followed with addition of 200 μl of 35 mM Ammonium Molybdate in 15 mM Zinc Acetate: 10% Ascorbic Acid (1:4) and incubated for an additional 20 minutes at 37°C. Additionally, 0.06 ml aliquots of potassium phosphate standards prepared in the buffer described above, were incubated in the plates containing the test and control substances, with SDS and detection reagent added. The liberation of inorganic phosphate was detected by its absorbance at 800 nm and quantitated by comparing the absorbance to a phosphate standard curve. The concentration dependence of the PGP was analyzed for evidence of saturation of PGP-ATPase activity, and apparent kinetic parameters were calculated by non-linear regression. The positive control for stimulation of ATPase activity was 20 μM verapamil, and the positive control for inhibition of basal ATPase activity was 25 mM ketoconazole.

[00072]   In a semi-quantiative assay for ATPase inhibition, Naltrexone, Naloxone and Nalmefene were hown to inhibit the ATPase associated with PGP1a as shown in Table 5.

Table 5: Vanadate-sensitive ATPase Activity

Concentration (μM)	Activity (nmol/mg min)		
	Naloxone	Naltrexone	Nalmefene
100	1.8	4.6	3.2
30	1.9	-	2.3
10	2	-	-

3	1.7	-	-
1	0.4	-	-

[00073]   The order of inhibition of the PgP1a associated ATPase was nalmefene, naltrexone and naloxone. Naloxone only weakly inhibited the PGP1a associated ATPase. None of the compounds were stimulators of ATPase.

Example 4 – Molecular Modeling of Opioid Analogues

[00074]   A molecular modeling analysis was performed on a series of compounds, including opioid analogues, to elucidate their mode of interaction with PARAGRAPH-1a, and to determine if possible, a pharmacophore for drug transporter inhibitors useful in the present invention. Exemplary compounds in this study were naltrexone, naloxone, nalmefene, 6-β-naltrexol and nalorphine. The structures of compounds are illustrated in Fig. 1. The compounds are structurally very similar, and exhibit two measured activities. "Activity 1" is characterized by a low capacity, high affinity binding site with activity ranging from 0.3 nM to greater than 200 μM. On the other hand, "activity 2" is characterized by a high capacity, low affinity binding site with activity ranging from 10 μM to greater than 100 μM. Table 6 provides the biological activities for each of the exemplary compounds.

Table 6: Biological Activity of Exemplary Compounds

Compound	Activity 1	Activity 2
Nalmefene	0.3 nM	100 μM
Naltrexone	0.3 nM	100 μM
Naloxone	1.0 nM	30 μM
6-β-Naltrexol	0.1 nM	100 μM
Nalorphine	N/A	N/A

[00075]   In performing the calculations for the molecular modeling analysis, two assumptions were made. First, nalorphine exhibits no measurable activity. Second, the structures of the compounds as represented in the Merck Index represent is the active form of the compound.

[00076]   An important difference in these compounds is that nalorphine lacks the hydroxyl group in the central ring at position 14 (*see, e.g.,* Figure 1), indicating that this hydroxyl group is a requirement for activity. The most active compounds (nalmefene and naltrexone) each have a hydrophobic group (cyclopropyl) tethered to the nitrogen,

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indicating that a hydrophobic moiety is partially responsible for the higher activity in these compounds. This moiety may be viewed as a necessary, but not sufficient condition, since several of the inactive compounds also possess this hydrophobic region. Initial activity data suggest that the electron density present at this location in naloxone (due to the ethylene substituent [C=C]) is contributory to its lower activity. The observation that 6-β-Naltrexol is even less active is attributed to the hydroxyl substituent at the 6 position being oriented β to the ring system, perhaps penetrating a sterically limited region in the receptor.

[00077]   In summary, the analysis indicates that the presence of the hydroxyl group at the 14-position may be required for activity, since nalorphine, with no measured activity, lacks this moiety. In addition, the two most active compounds (nalmefene and naltrexone) possess an ethylene group and a carbonyl group respectively at the 6-position. This may represent a requirement for electron density at this position, rather than a hydrogen-bond acceptor site, as there is only a one order of magnitude difference in activity (0.3nM vs. 3nM) between the ethylene group (nalmefene) and the carbonyl group (naltrexone). There is a potential steric limit for substituent size or directionality at the 6-position, based on the analysis of 6-β-Naltrexol indicates that its hydroxyl group in a direction that penetrates into this region. Finally, a hydrophobic group is required as the N-substituent for highest activity, as naloxone, with a double bond rather than the cyclopropyl group exhibits significantly lower activity.

[00078]   When the novel analysis described above is now considered in conjunction with a recent scientific article investigated the ability of a variety of peptidomimetic thrombin inhibitors to inhibit intestinal transport [Kamm et al., "Transport of peptidomimetic thrombin inhibitors with a 3-amino-phenylalanine structure: permeability and efflux mechanism in monolayers of a human intestinal cell line (Caco-2)." Pharm. Res. 18:1110-8 (2001)], it is possible to utilize additional structural information from Kamm to develop a model of interaction with PGP. Kamm et al. proposed that basic and acidic residues of amidino-phenylalanine-derived thrombin inhibitors mediate affinity to intestinal efflux pumps, presumably PGP and MRP. Structural information from Kamm et al. useful in the novel QSAR analysis of the present invention is summarized below:
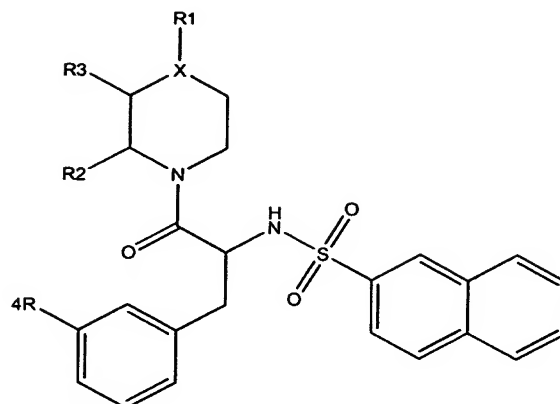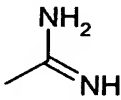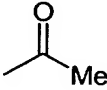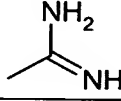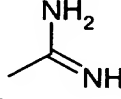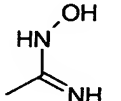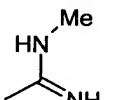
Table 7: R-groups of compounds Kamm et al.

Structure	R1	R2	R3	X	R4
1	Me	H	H	C	
2	H	COOH	H	C	
3	H	COO-Me	H	C	
4	H	H	COOH	C	
5	H	H	COO-Me	C	
6	COOH	H	H	C	
7	COO-Me	H	H	C	
8	COOH	H	H	C	
9	COOH	H	H	C	

Structure	R1	R2	R3	X	R4
10	H	H	H	N	NH_2 / NH (amidine)
11	C(=O)Me (acetyl)	H	H	N	NH_2 / NH (amidine)
(12)	Me	H	H	C	NH_2 / NH (amidine)
13	Me	H	H	C	NH_2
14	Me	H	H	C	$-CH_2NH_2$
15	Me	H	H	C	HN-OH / NH (hydroxyamidine)
16	Me	H	H	C	HN-Me / NH (methylamidine)

[00079]   The intestinal permeability coefficients of the Kamm compounds were studied using Caco-2 monolayers and reverse-phase HPLC method for quantitation. Further the efflux ratios (transport from B to A:transport from A to B) were calculated. The efflux ratios for a selection of the Kamm compounds measured at 250 μM are provided in Table 8.

Table 8: Efflux Ratios at 250 μM

Structure	Efflux Ratio B→A/A→B
1	45.0
2	2.8
3	10.5
4	2.7
5	11.1
6	1.9
7	6.0

8	22.1
9	1.1
10	0.8
11	2.4

[00080]   The efflux ratios the remaining Kamm compounds measured at 100 μM are provided in Table 9.

Table 9: Efflux Ratios at 100 μM

Structure	Efflux Ratio $B{\rightarrow}A/A{\rightarrow}B$
1	16.3
12	24.9
13	1.14
14	3.43
15	1.31
16	13.0

[00081]   Comparable measurements for the opioid analogues are provided in Table 10. The data of Table 10 was obtained from the experiments described in Example 1. Efflux ratios normalized to 25 μM ketoconazole (Keto) are presented in parentheses after the measured ratios.

Table 10: Efflux Ratios of Opioid Analogues

Structure	Keto @25μM	Hi Affinity / Low Cap		Low Affinity / Hi Cap	
		[C] μM	B A/A B	[C] μM	B A/A B
Nalmefene	1.4	0.0003	4.2 (3.0)	100	2.6 (1.9)
Naltrexone	1.0	0.0003	3.5 (3.5)	100	2.7 (2.7)
Naloxone	1.1	0.001	3.4 (3.1)	30	2.6 (2.4)
Naloxone				100	2.7 (2.5)
6-β-Naltrexol	1.0	0.0001	4.4 (4.4)	100	3.4 (3.4)

[00082]   An overlay of the opioid analogue structures is presented in Fig. 2. All active ("Activity 1") compounds share the following features: two hydroxyl groups (a) at positions

3 and 14, a furan ring system, a hydrophobic region in ring system, a region of electron density at position 6 (**b**), and a cyclic tertiary nitrogen (**c**) with an appended hydrophobic group (**d**).

[00083]   Molecular Orbital calculations were performed on the compounds using Spartan (Wavefunction, Inc.). There were no appreciable differences among the active compounds with respect to their electrostatic potentials. The electrostatic potential of nalmefene and naloxone are illustrated in FIGS. 3A and B respectively. The arrows indicate the hydroxyl group hydrogen-bond donor sites noted above.

[00084]   Two views of an overlay of nalmefene and the low energy conformer of Kamm Compound 1 was prepared. The ring stacking structure predicted by Confort for the Kamm compounds embodies a conserved hydrophobic region shared by the both the Kamm compounds and the exemplary opioid compounds. The hydrogen-bond donor sites noted in the FIG. 3 are overlap the predicted hydrogen bonding sites of the Kamm compound. The nalmefene furan ring oxygen overlays on an aromatic ring in Kamm Compound 1, suggesting that the oxygen atom is not necessary for this activity.

[00085]   *In silico* analyses of chemical compounds were conducted as follows: Diversity estimations were made on nalmefene, naloxone, naltrexone, 6-β-naltrexol, and the 16 Kamm et al structures using DiverseSolutions software from Tripos (R.S. Pearlman, UT-Austin). A chemistry space defined by approximately 900,000 chemical entities (several commercially available databases of compounds) was used as a reference. The commercial databases used as sources of the 900,000 chemical entities were MDL Information Systems (http://www.mdli.com), ACD Database (http://www.mdli.com/cgi/dynamic/product.html?uid=$uid&key=$key&id=17), NCI (http://dtp.nci.nih.gov/docs/3d_database/structural_information/smiles_strings.html), Aldrich (http://www.sigma-aldrich.com/saws.nsf/home?openframeset), ASINEx Ltd. (http://www.asinex.com), and Chemstar (http://www.chemstar.ru). A transporter-relevant subspace was determined based on the former chemistry space, using the "B A / A B" efflux ratios to represent the activities. In order to have sufficient data, the Kamm et al data was combined with the high affinity/low capacity data provided for the exemplary opioid compounds. The 200 "nearest neighbors" are listed in Table 11 below. Note that in the Receptor-Relevant Subspace, the active compounds are focused in a small region of the overall chemistry space.

Table 11: 200 Nearest Neighbors

Rank	Database I.D. #	Distance to Exemplary compound		
1	70413	0.0096	to	Naloxone
2	MFCD00133650	0.0184	to	Nalmefene
3	349115	0.4061	to	Nalmefene
4	BAS 3387173	0.5101	to	Naloxone
5	BAS 1002455	0.5195	to	Naloxone
6	BAS 3387155	0.5243	to	Naloxone
7	BAS 1268016	0.5345	to	Naloxone
8	BAS 3387156	0.5412	to	Naloxone
9	BAS 3387130	0.5462	to	Naloxone
10	MFCD01935543	0.5507	to	Naloxone
11	688277	0.5913	to	6-β-Naltrexol
12	BAS 1002441	0.6179	to	Naloxone
13	BAS 3386059	0.6369	to	Naloxone
14	BAS 1003176	0.6370	to	Naloxone
15	BAS 1004848	0.6434	to	Naloxone
16	MFCD00273259	0.6436	to	Nalmefene
17	MFCD00273270	0.6458	to	Naloxone
18	MFCD00273266	0.6482	to	Naloxone
19	BAS 3386023	0.6526	to	Naloxone
20	BAS 2026128	0.6569	to	Naloxone
21	617005	0.6581	to	6-β-Naltrexol
22	MFCD00079194	0.6622	to	6-β-Naltrexol
23	19045	0.6665	to	6-β-Naltrexol
24	76021	0.6733	to	Nalmefene
25	BAS 1002442	0.6770	to	Naloxone
26	MFCD00271723	0.6822	to	Naloxone
27	MFCD00273273	0.6884	to	Nalmefene
28	MFCD00273264	0.6968	to	Nalmefene
29	BAS 2026145	0.6977	to	Naloxone
30	BAS 3387114	0.7036	to	Naloxone
31	376679	0.7051	to	Naltrexone

Rank	Database I.D. #	Distance to Exemplary compound		
32	379963	0.7051	to	Naltrexone
33	157870	0.7144	to	Nalmefene
34	MFCD00273274	0.7198	to	Naloxone
35	MFCD00273260	0.7228	to	Nalmefene
36	BAS 1003163	0.7272	to	Naloxone
37	BAS 1003182	0.7388	to	Naltrexone
38	BAS 0510629	0.7564	to	Naltrexone
39	BAS 1002419	0.7571	to	Naloxone
40	18579	0.7600	to	Nalmefene
41	58796	0.7600	to	Nalmefene
42	BAS 1004835	0.7634	to	Naloxone
43	BAS 2004373	0.7646	to	Naloxone
44	693856	0.7680	to	Nalmefene
45	MFCD01764789	0.7687	to	Naloxone
46	MFCD00271738	0.7719	to	Nalmefene
47	BAS 2025996	0.7741	to	Naloxone
48	BAS 2282169	0.7798	to	Nalmefene
49	MFCD00273268	0.7895	to	Naloxone
50	MFCD00179880	0.7997	to	Naloxone
51	BAS 1507170	0.8014	to	Nalmefene
52	BAS 3386088	0.8017	to	Naloxone
53	MFCD00272082	0.8183	to	Nalmefene
54	MFCD00271113	0.8289	to	6-β-Naltrexol
55	116054	0.8308	to	6-β-Naltrexol
56	BAS 1004837	0.8352	to	Naloxone
57	134536	0.8364	to	6-β-Naltrexol
58	615801	0.8556	to	Naltrexone
59	404374	0.8695	to	Nalmefene
60	MFCD00273318	0.8697	to	Nalmefene
61	MFCD00271094	0.8774	to	Nalmefene
62	202587	0.8895	to	Nalmefene

Rank	Database I.D. #	Distance to Exemplary compound		
63	693862	0.8919	to	Nalmefene
64	MFCD00467140	0.9049	to	Nalmefene
65	693863	0.9093	to	Naltrexone
66	MFCD00271196	0.9123	to	Nalmefene
67	BAS 3386092	0.9195	to	Naloxone
68	693855	0.9235	to	Nalmefene
69	BAS 3386091	0.9278	to	Naloxone
70	MFCD00665833	0.9291	to	Naltrexone
71	404368	0.9412	to	6-β-Naltrexol
72	BAS 0606820	0.9478	to	Naloxone
73	693859	0.9485	to	Nalmefene
74	BAS 0436353	0.9653	to	Naloxone
75	MFCD00167445	0.9681	to	Naltrexone
76	MFCD00667402	0.9742	to	Nalmefene
77	MFCD002258126	0.9767	to	Naloxone
78	MFCD00143186	0.9850	to	Naltrexone
79	119887	0.9932	to	Naloxone
80	404365	1.0016	to	Nalmefene
81	MFCD01871411	1.0116	to	Naloxone
82	152720	1.0147	to	6-β-Naltrexol
83	117581	1.0164	to	Naloxone
84	669466	1.0171	to	Naloxone
85	MFCD00271129	1.0287	to	Nalmefene
86	689431	1.0350	to	6-β-Naltrexol
87	MFCD00056772	1.0390	to	Nalmefene
88	MFCD00199295	1.0449	to	Nalmefene
89	R191469	1.0457	to	Nalmefene
90	375504	1.0503	to	Naloxone
91	692397	1.0656	to	Naloxone
92	MFCD00433684	1.0691	to	Naloxone
93	693860	1.0709	to	Nalmefene

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Rank	Database I.D. #	Distance to Exemplary compound		
94	MFCD01764791	1.0725	to	Naloxone
95	BAS 1519270	1.0776	to	Naloxone
96	BAS 3385849	1.0828	to	Naloxone
97	MFCD00673308	1.0866	to	Nalmefene
98	404356	1.0990	to	Nalmefene
99	43938	1.1067	to	Nalmefene
100	117181	1.1092	to	Naltrexone
101	MFCD00094379	1.1109	to	Nalmefene
102	404369	1.1109	to	6-β-Naltrexol
103	381577	1.1111	to	Naloxone
104	S842214	1.1117	to	Nalmefene
105	134602	1.1123	to	6-β-Naltrexol
108	CHS 0316796	1.1130	to	Naloxone
107	134604	1.1147	to	Nalmefene
108	R171697	1.1334	to	Nalmefene
109	MFCD00667401	1.1343	to	Nalmefene
110	S959863	1.1367	to	6-β-Naltrexol
111	35545	1.1369	to	6-β-Naltrexol
112	134598	1.1369	to	6-β-Naltrexol
113	S310778	1.1403	to	Naloxone
114	669800	1.1408	to	Naloxone
115	BAS 0083962	1.1413	to	Naltrexone
116	MFCD01765597	1.1424	to	6-β-Naltrexol
117	682334	1.1427	to	Naloxone
118	BAS 0631739	1.1428	to	Nalmefene
119	MFCD00144882	1.1486	to	6-β-Naltrexol
120	MFCD00229975	1.1497	to	Naloxone
121	R171700	1.1568	to	Nalmefene
122	134592	1.1633	to	6-β-Naltrexol
123	401210	1.1662	to	Nalmefene
124	BAS 2026074	1.1715	to	Naltrexone

Rank	Database I.D. #	Distance to Exemplary compound		
125	BAS 3050727	1.1767	to	Nalmefene
126	BAS 0341630	1.1851	to	Naloxone
127	97817	1.1901	to	Naloxone
128	ASN 3185453	1.1958	to	Naloxone
129	21257	1.1962	to	6-β-Naltrexol
130	134601	1.2005	to	6-β-Naltrexol
131	BAS 2026075	1.2027	to	6-β-Naltrexol
132	BAS 1996620	1.2114	to	6-β-Naltrexol
133	MFCD01314356	1.2147	to	Naloxone
134	BAS 2026097	1.2207	to	Naltrexone
135	BAS 1914007	1.2210	to	Naloxone
136	CHS 0003221	1.2266	to	Naloxone
137	667258	1.2274	to	Naloxone
138	37625	1.2351	to	Nalmefene
139	BAS 1003093	1.2362	to	6-β-Naltrexol
140	16468	1.2380	to	Naloxone
141	CHS 0227049	1.2409	to	Naloxone
142	BAS 0315050	1.2410	to	Nalmefene
143	BAS 1289763	1.2421	to	Naloxone
144	349127	1.2429	to	Naloxone
145	635928	1.2496	to	Nalmefene
146	BAS 2377555	1.2507	to	6-β-Naltrexol
147	MFCD00665835	1.2508	to	Naltrexone
148	47931	1.2547	to	6-β-Naltrexol
149	76435	1.2572	to	Nalmefene
150	90558	1.2581	to	Naloxone
151	MFCD00206273	1.2608	to	Naloxone
152	159208	1.2670	to	Nalmefene
153	BAS 0341580	1.2672	to	Naltrexone
154	BAS 2377575	1.2678	to	Naltrexone
155	MFCD01765638	1.2681	to	Nalmefene

Rank	Database I.D. #	Distance to Exemplary compound		
156	R171484	1.2684	to	Nalmefene
157	700350	1.2716	to	Naloxone
158	16907	1.2740	to	Nalmefene
159	R170623	1.2754	to	Nalmefene
160	S98907	1.2776	to	Naloxone
161	10464	1.2777	to	Naloxone
162	215214	1.2777	to	Naloxone
163	R171425	1.2802	to	Nalmefene
164	MFCD00153032	1.2831	to	6-β-Naltrexol
165	S196991	1.2850	to	Naltrexone
166	R170291	1.2863	to	Naloxone
167	682335	1.2867	to	Naloxone
168	UFCD00667377	1.2889	to	Nalmefene
169	106242	12944	to	Naloxone
170	R170410	1.2989	to	Naloxone
171	MFCD0005912	1.2996	to	Naloxone
172	MFCD01765637	1.3018	to	Nalmefene
173	376678	1.3028	to	Naltrexone
174	MFCD01314431	1.3031	to	Naloxone
175	370278	1.3040	to	Nalmefene
176	MFCD00242635	1.3054	to	6-β-Naltrexol
177	S602965	1.3058	to	Naltrexone
178	370279	1.3063	to	Nalmefene
179	157877	1.3099	to	Nalmefene
180	19046	1.3103	to	6-β-Naltrexol
181	117862	1.3103	to	6-β-Naltrexol
182	MFCD00667305	1.3134	to	Nalmefene
183	MFCD00667382	1.3161	to	Nalmefene
184	611276	1.3178	to	6-β-Naltrexol
185	BAS 1099232	1.3197	to	Naltrexone
186	BAS 0313319	1.3206	to	6-β-Naltrexol

Rank	Database I.D. #	Distance to Exemplary compound		
187	401211	1.3254	to	Nalmefene
188	409635	1.3263	to	Nalmefene
189	106231	1.3271	to	Naloxone
190	375505	1.3289	to	Naloxone
191	BAS 1053035	1.3309	to	Naloxone
192	ASN 3160807	1.3316	to	Naloxone
193	324633	1.3331	to	Naloxone
194	370277	1.3392	to	Naloxone
195	MFCD00375811	1.3428	to	6-β-Naltrexol
196	CHS 0305736	1.3435	to	6-β-Naltrexol
197	BAS 0659522	1.3435	to	6-β-Naltrexol
198	381576	1.3461	to	Naloxone
199	CHS 0120289	1.3484	to	Naloxone
200	351159	1.3490	to	Nalmefene

[00086]   The distance between the hydroxyl groups in the pharmacophore ("H" of OH to "H" of OH) is approximately 7.4 Å. The equivalent distance in "Kamm 1" is ~7.7 Å. These distances are to the Hydrogen atoms, rather than the H-bond acceptors in the binding site. The N-substituent lengths of nalmefene (from N to terminal Carbons) are ~3.9 Å and ~3.5 Å. N-substituent length of naloxone (from N to terminal Carbon) is ~3.4 Å.

[00087]   The three-dimensional coordinates of naltrexone are provided in Table 12.

Table 12: Three-Dimensional Coordinates

ATOM	X	Y	Z	Type	Charge
C1	-0.0352	-0.1951	0.0725	C.ar	0.1489
C2	2.0834	-0.0915	0.6474	C.3	0.1387
C3	2.3288	1.3986	0.5409	C.2	0.1298
C4	2.7343	2.1393	1.7840	C.3	0.0249
C5	1.6213	1.9380	2.8395	C.3	-0.0154
C6	1.5391	0.4338	3.2099	C.3	0.0664
C7	1.2934	-0.4401	1.9514	C.3	0.0294
C8	0.3791	0.1181	4.2040	C.3	0.0429
C9	-1.0383	0.5073	3.6641	C.3	0.0052
C10	-1.2030	0.2284	2.1659	C.ar	-0.0334
C11	-0.0782	-0.1163	1.4337	C.ar	-0.0151
C12	-2.4171	0.3074	1.4505	C.ar	-0.0499
C13	-2.4130	0.2019	0.0328	C.ar	-0.0203
C14	-1.2074	0.0000	-0.6793	C.ar	0.1404
O15	1.2170	-0.4755	-0.4637	O.3	-0.2867
C16	1.3253	-1.9545	2.2801	C.3	-0.0592

N17	0.4895	-1.3246	4.5611	N.3	-0.2960
C18	0.3363	-2.2765	3.4315	C.3	-0.0091
O19	2.8028	0.1380	3.8337	O.3	-0.3969
O20	-1.1968	0.0000	-2.0760	O.3	0.3351
O21	2.1919	2.0008	-0.5126	O.2	-0.3894
C22	-0.1632	-1.7771	5.8169	C.3	0.0022
C23	0.2667	-0.9142	7.0296	C.3	-0.0282
C24	-0.5945	-1.0908	8.2998	C.3	-0.0488
C25	-0.7018	0.2063	7.4700	C.3	-0.0488
H26	-3.3439	0.2757	-0.5190	H	0.0719
H27	-3.3515	0.4481	1.9839	H	0.0519
H28	-0.7033	-2.2458	3.0686	H	0.0417
H29	0.5379	-3.3100	3.7583	H	0.0417
H30	1.0537	-2.5464	1.3901	H	0.0165
H31	2.3491	-2.2448	2.5610	H	0.0165
H32	3.7066	1.7640	2.1382	H	0.0495
H33	2.8430	3.2119	1.5551	H	0.0495
H34	0.6739	2.3152	2.4251	H	0.0308
H35	1.8585	2.5217	3.7437	H	0.0308
H36	-1.2074	1.5867	3.7999	H	0.0488
H37	-1.8236	-0.0234	4.2195	H	0.0488
H38	3.0581	-0.5987	0.5948	H	0.0780
H39	0.5866	0.7227	5.1003	H	0.0510
H40	-0.3069	0.0000	-2.4176	H	0.2424
H41	2.8163	-0.7158	4.2555	H	0.2089
H42	0.1871	-2.7925	6.0602	H	0.0429
H43	-1.2569	-1.8218	5.7021	H	0.0429
H44	1.3391	-0.7446	7.2194	H	0.0313
H45	-1.6257	0.3467	6.8884	H	0.0268
H46	-0.2477	1.1098	7.9059	H	0.0268
H47	-1.4559	-1.7752	8.2529	H	0.0268
H48	-0.0805	-1.0045	9.2699	H	0.0268

[00088]   Through the use of these coordinates a pharmacophore may be defined by: (1) a hydrogen bonding moiety at a three-dimensional location corresponding to the hydroxyl at position 3 of naltrexone; (2) a hydrogen bonding moiety at a three-dimensional location corresponding to the hydroxyl at position 14 of naltrexone; (3) a hydrophobic moiety at a three-dimensional location corresponding to the cyclopropyl moiety appended to the nitrogen of naltrexone; and (4) a region of electron density at a three-dimensional location corresponding to the ethylene moiety at 6-position of naltrexone.

[00089]   All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication of patent application was specifically and individually indicated to be incorporated by reference.

[00090]   The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.